Building functional surfaces for biosensors development

Carlos Morón , Alfonso Garcia , Enrique Tremps
Jose Andrés Somolinos

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Abstract. Polyelectrolyte multilayers (PEM) built by layer-by-layer technique have been extensively studied over the last years, resulting in a wide variety of current and potential applications. This technique can be used to construct thin films with different functionalities, or to functionalize surfaces with substantial different properties of those of the underlying substrates. The multilayering process is achieved by the alternate adsorption of oppositely charged polyelectrolytes. In this work we get advantage of the protein resistant property of the Poly(l-lysine)-graft-(polyethyleneglycol) to create protein patterns. Proteins can be immobilized on a surface by unspecific physical adsorption, covalent binding or through specific interactions. The first protein used in this work was laccase, a copper-containing redox enzyme that catalyse the oxidation of a broad range of polyphenols and aromatic substrates, coupled to the reduction of O_2 to H_2O without need of cofactors. Applications of laccases have been reported in food, pulp, paper, and textile industry, and also in biosensor development. Some uses require the immobilization of the enzyme on solid supports by adsorption, covalent attachment, entrapment, etc, on several substrates. Especially for biosensor development, highly active, stable and reproducible immobilization of laccase is required.

Introduction

Different parameters influence the building-up of the polyelectrolyte multilayer, such as polymer molecular weight and concentration, ionic strength, surface and polymer charge density [1; 5]. But once the building conditions are set, the PEM assembly is highly reproducible. One of the biggest advantages of polyelectrolytes (PEs) technology is the versatility. PE can be applied on a wide variety of substrates and different surfaces characteristics can be obtained depending on the PEs used [2; 3]. Integrating proteins on/or in the PE multilayer, different functionalities ranging from DNA to specific recognition entities, can be incorporated to the surface [4; 6].

Besides, by means of a soft-lithographic technique such as microcontact printing, it is possible to obtain surfaces where a PE is deposited following a desired feature with micrometers dimensions on a opposite charge PE surface [7].

Covalent protein immobilisation often begins with surface modification and/or an activation step. Two activation steps commonly used are based on carboxylic acid or amine moieties that through the reaction with coupling agents such as glutaradehyde or carbodiimide, chemically anchors the biomolecule though its amine groups [8].

Also, the use of biotin-strept(avidin) system is an example of specific interaction systems that can be used as bridge between a surface and the biomolecule of interest. The highly specific interaction between biotin and strept(avidin), and feasibility to introduce biotin in a target biomolecule without affecting the activity, lead to other possible method to modify surfaces through biotynalated molecules.

Each of the methods offers advantages and disadvantages that depend on several factors. In general, chemical immobilisation methods tend to disturb the native structure of the protein due to the covalent bonds formed as a result of immobilisation. By contrast, such covalent linkages

provide strong stable attachment. However, the adsorption typically perturb the protein structure much less depending on its rigidity [8].

The protein used in this work was laccase, a copper-containing redox enzyme that catalyse the oxidation of a broad range of polyphenols and aromatic substrates, coupled to the reduction of O_2 to H_2O without need of cofactors. Especially for biosensor development, highly active, stable and reproducible immobilisation of laccase is required. Different configuration for laccase immobilization on polyelectrolyte multilayers, self assembly monolayers have been proposed.

Experimental and results

Polyelectrolyte multilayer assembly

The assembly of the polyelectrolyte multilayers was monitored by Quartz crystal microbalance with dissipation monitoring (QCM-D). The change in frequency after each layer is showed in figure 1. Poly(ethylenimine) (PEI) was the first layer with a $\Delta f = 10 \pm 2$ Hz and $\Delta D = 0.1 \pm 0.3x10\text{-}6$ after adsorption. Lately, PSS (Poly(sodium 4-styrenesulfonate)) and PAH (Poly(allylamine hydrochloride)) were adsorbed until a total of 4 layers.

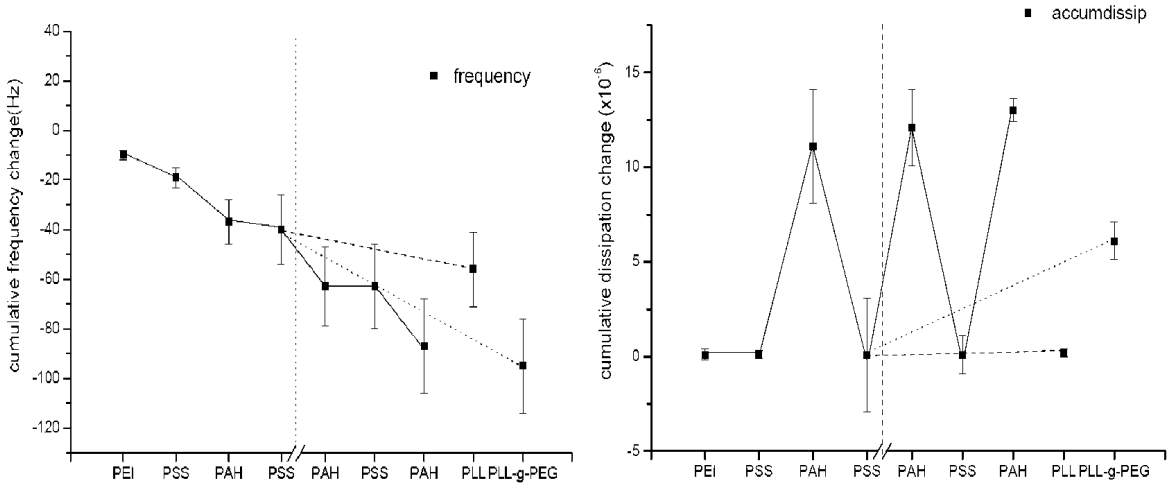

Fig. 1. Frequency and dissipation chance during the layer-by-layer deposition on gold crystals. A continuous decrease in frequency was observed after deposition of every PE layer. The dissipation increased after PAH adsorption, but always decreased after PSS adsorption.

The change in frequency for PSS after PEI was of 9 ± 2 Hz and the dissipation remained close to 0. When the first PAH layer was adsorbed on PSS, the change of frequency was of 18 ± 5 Hz, and its deposition always led to an increase in dissipation of $10 \pm 3x10^{-6}$. After the second layer of PSS, the frequency remained almost constant, with a $\Delta f = 3 \pm 2$ Hz and a $\Delta D = -10 \pm 3x10^{-6}$. This behaviour repeats for the next PAH/PSS/PAH adsorption. After the second PSS layer, other 2 positive polyelectrolytes were adsorbed in different experiments, PLL and PLL-g-PEG (Poly(l-lysine)-graft-(polyethyleneglycol)). After PLL, the frequency drop was of 16 ± 1 Hz and the dissipation increase $0.1 \pm 0.2x10^{-6}$. The PLL-g-PEG adsorption led to the biggest change in frequency for only one layer, 55 ± 5 Hz, and the dissipation increase was $6 \pm 1x10^{-6}$.

Contact angle

The contact angle of the polyelectrolyte surfaces was measured and the values obtained are presented in table 1 and 2. Silicon wafers and glass were cleaned with the RCA method, making the surface hydrophilic with a contact angle of 12 ± 6^{o}. The deposition of PEI made the surface less hydrophilic, with a contact angle of 48 ± 22^{o}. After the deposition of a PSS/PAH/PSS, the contact angle was measured again. The contact angle of PSS and PAH were similar on SiOx, but slightly

lower when measured on glass. The PLL presented a contact angle of 31±3° on glass, and as expected the PLL-g-PEG surface presented a most hydrophilic surface, with a contact angle of 24 ±3°.

Table 1. PE contact angle measurements on SiOx.

Surface	*Contact angle*
SiOx	12 ± 6
PEI	48 ± 22
PSS	44 ± 5
PAH	44 ± 9
PLL-g-PEG	24 ± 3

Table 2. PE contact angle measurements on glass.

Surface	*Contact angle*
PSS	33 ± 5
PAH	38 ± 2
PLL	31 ± 3

Polyelectrolyte patterning

The patterning technique used was the micro-contact printing. It was possible to obtain different patterning features with different polyelectrolytes. Figure 2 shows some examples obtained with fluorescently labelled PAH (PAH-FITC). Besides, PLL-g-PEG patterns were obtained. Backfilling of the pattern for PLL-g-PEG pattern with PAH and PLL were accomplished. In figure 2, RBITC-PAH was used to backfill the free PSS areas between the PLL-g-PEG stripes.

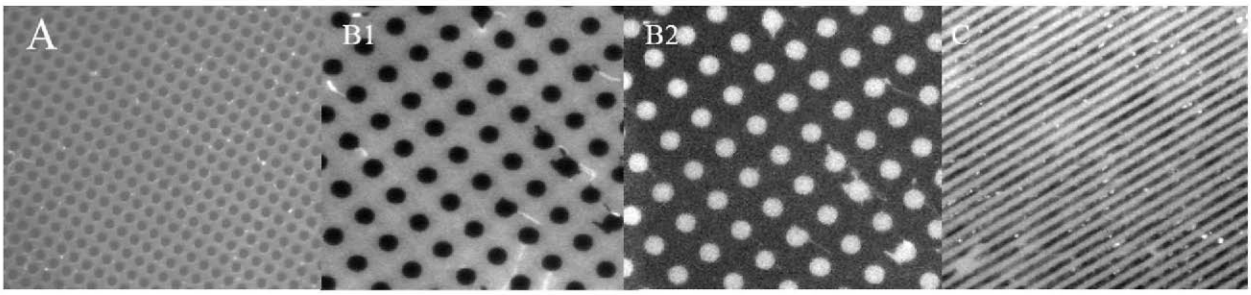

Fig. 2. Fluorescence microscope images of patterned surfaces. A) PAH-FITC is the complementary area of circles D=10 μm, B1) RBITC-PAH stamped pattern and B2) FITC-PAH pattern obtained after backfilling the RIBTC-PAH free areas, C) PLL-g-PEG pattern backfilled with RBITC-PAH..

Laccase immobilization and interaction with polyelectrolytes

Laccase was covalently immobilized with glutaraldehyde (GA). GA first reacted with the amines of poly(ethyleneimine) (PEI) adsorbed on gold, and later with laccase. The enzyme immobilization process was followed by QCM-D as shown in figure 3. The adsorption of PEI and the reaction with GA led to a compact layer with Δf and ΔD change of 10±2 Hz and 0.1 ± 0.3x10^{-6}, and 6±1 Hz and 0 ± 0.3x10^{-6}, respectively.

Due to the fact that succinate buffer was used to dilute the laccase, prior to laccase immobilization, this buffer was pumped through the QCM chamber. Before dilution, the crude extract was centrifuged and filtered as explained in Materials and Methods. The protein concentration and activity of the diluted laccase were 270±20 mgl^{-1} and 570±80 Ul^{-1}. Laccase solution was injected in the chamber and after stabilization, the change in frequency and dissipation were of 24±5 Hz and 1.2 ± 0.6x10^{-6}.

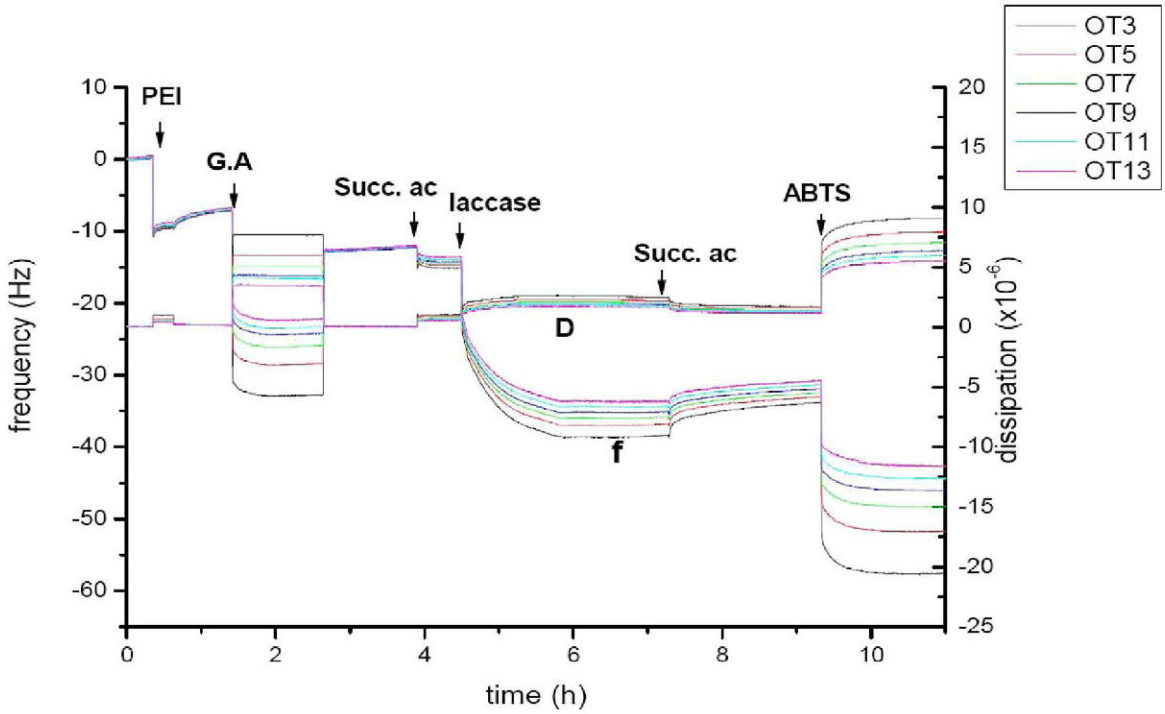

Fig.3. QCM-D measurement of the laccase immobilization. A decrease in frequency without significant change in dissipation was observed after adsorption of PEI and the covalent attachment of GA. The immobilization of laccase led to a decrease in frequency of 24 Hz with dissipation increase of 1.2×10^{-6}.

In order to check whether the change in frequency was due to immobilization of active laccase, 2,2'-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) was injected into the chamber and while recording frequency and dissipation change, the effluent was collected. After injecting succinate buffer solution to remove ABTS, the frequency and dissipation returned to their original values within the experimental error. Since laccase oxidises ABTS to a dark green product, the presence of active laccase was confirmed by measuring an increase in the absorbance of the effluent. When the flow increased, the effluent absorbance decreased. After laccase immobilization, polyelectrolytes were adsorbed. After the adsorption of PAH, PAH/PSS/PAH, and $(PAH/PSS)_2$, ABTS was pumped into the chamber to test if the enzyme was still active. We have observed that the PE on the laccase changed the shape of the adsorption curve.

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